Ex vivo expansion of haematopoietic progenitors on an endothelialized hydroxyapatite matrix

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Autologous haematopoietic progenitor cell (HPC) transplantation is increasingly used to restore haematopoiesis after high-dose chemotherapy treatments. The present study was designed to analyse the ability of hydroxyapatite (HAP) seeded with endothelial cells (EC) to support the proliferation and differentiation of $CD34^+$ HPC in static culture conditions. HAP is endothelializable as assessed by scanning electron microscopy and time-course DNA synthesis analysis using tritiated thymidine incorporated in EC isolated from human umbilical vein cord. Short-term coculture experiments in which $CD34^+$ cells isolated from human cord blood were seeded on endothelialized HAP, were performed. Results show that endothelialized HAP is permissive to $CD34^+$ cell expansion with a maximum expansion obtained between days 7 and 14 of coculture in the presence of IL-1 and IL-3 when compared with other experiments omitting either EC or interleukins. From morphological analyses, the expanded cell population mainly belonged to the myelocytic lineage with 33% mature cells (polymorphonuclear neutrophils and monocytes) at day 14 of coculture. The immature HPC could remain trapped within HAP while giving rise to a more mature progeny that exit from HAP microenvironment.

1. Introduction

Autologous progenitor cell transplantation is now increasingly used to restore haematopoiesis after high-dose chemotherapy in the treatment of solid tumor or haematological malignancies. Haematopoietic progenitor cells (HPC) can be collected from the peripheral blood after mobilization with haematopoietic growth factors and the transplantation with such cells may reduce the duration of granulocytopenia and thrombocytopenia when compared with bone marrow progenitor cells [1]. Attention is currently being directed at techniques aimed at expanding the pool of HPC. A number of haematopoietic growth factors have been identified that promote the expansion and the differentiation of HPC [2] and recent reports indicate that *ex vivo* expanded $CD34^+$ cells can supply a population of HPC that have the ability to restore sustained haematopoiesis in high-dose chemotherapy-treated patients [3]. Comparison of stromal cells/haematopoietic progenitors cocultures with multicytokine stroma-free expansion systems indicated that optimal *ex vivo* expansion of the HPC requires stromal cell-derived growth promoting factors, particularly for the expansion of the most primitive cells [4]. Bioreactors using stromalized biomaterials have been developed [5]. The role of endothelial cells (EC) as feeder cells for the expansion of HPC has been recently underlined [6, 7]. Hydroxyapatite (HAP) is a calcium phosphate, the composition of which is very close to the mineral part of calcified tissues. It has been shown to be cellularizable by whole bone marrow [8] or osteoprogenitor cells isolated from human bone marrow [9]. Its particular three-dimensional lattice structure and its osteoinductive properties make it of potential interest to obtain a "hybrid bone" with haematopoietic activity [10]. The aim of our study was to analyse the role of endothelialized HAP in supporting the proliferation and differentiation of HPC.

2. Materials and methods

Synthetic HAP was manufactured by Bioland (Toulouse, France). All samples were cylindrically shaped (3 mm height, 6 mm diameter) and heat sterilized. Two types of HAP porosity were used, 400–700 (HAP1) and 800–1000 μm (HAP2). Human umbilical vein endothelial cells (HUVEC) were isolated and characterized as previously described [11] and used at the first passage. Cells from three donors were pooled together for each experiment.

$CD34^+$ progenitor cells ($CD34^+$ cells) were isolated from cord blood with CD34 Progenitor Cell Isolation

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kit (Qbend/10; Miltenyi Biotec, TEBU, France) after mononuclear cell separation with Ficoll-Hypaque (1.077 g ml^{-1}; Eurobio, France). The purity of the CD34$^+$ cell population was assessed by flow cytometry analysis using an anti-CD34$^+$ phycoerythrin-conjugated monoclonal antibody (Anti-HPC A2-2; Becton Dickinson, France) and viability by Trypan blue exclusion.

2.1. Endothelialization of HAP

HUVEC were seeded on HAP coated with either bovine serum albumin (BSA, 1% wt/vol) or collagen IV (coll IV, 10 μg ml^{-1}) or fibronectin (Fn, 10 μg ml^{-1}) or gelatin (1% wt/vol), or uncoated. Controls were coverslips identically coated. After coating, samples were dried 24 h at room temperature. Before cell seeding, HAP samples were placed on agarose [12] (4%, wt/vol) in 24-well plastic plates (Nunc, Roskilde, Denmark). HUVEC seeding concentration was 4×10^6 cells/ml, with 10 μl being seeded on each dried HAP sample. HUVEC were allowed to adhere 20–30 min at 37 °C. Iscove Modified Dulbecco's Medium (IMDM), 20% Foetal Calf Serum (FCS), 90 μg ml^{-1} heparin, 20 μg ml^{-1} Endothelial Cell Growth Supplement was then carefully added into the wells. A second HUVEC seeding was done the next day. After 7 days culture, this medium was replaced by CD34$^+$ cell culture medium (see below). DNA synthesis on gelatin-coated HAP2 samples was evaluated with [^{3}H] thymidine incorporation [13]. Controls were performed using unseeded gelatin-coated HAP2 samples. Four samples were tested for each point.

Scanning electron microscopy (SEM) was performed at days 7, 14, 21 using a standard protocol [13]: briefly, cells were fixed then washed and dehydrated. The dehydrated samples were critically point dried and finally coated with a gold–palladium target before observation. SEM was conducted both to appreciate the influence of different coatings on the quality of HUVEC covering and to corroborate the results of cell proliferation evaluation obtained on gelatin-coated HAP2.

2.2. HPC expansion on endothelialized HAP

Some 5×10^3 CD34$^+$ cells (90%–95% purity, 100% viability) were seeded on the endothelialized HAP in IMDM, 20% FCS, 100 IU ml^{-1} IL-1, 50 ng ml^{-1} IL-3, and grown in static culture conditions. All the non-adherent cells exited from the HAP samples were removed by pipetting on days 7, 14, 21 of coculture, and the total medium was changed. Cells were counted, their viability assessed and fold expansion values were calculated by dividing cell numbers obtained at previous cited days by the seeded cell number (5×10^3). For morphological analysis, cytospin preparations were stained with May-Grunwald Giemsa according to standard procedures [11] and SEM was performed.

3. Results

3.1. HAP is endothelializable

HUVEC did not attach to uncoated or BSA-coated HAP. No difference was seen between Fn, coll IV and gelatin coatings for the quality of endothelialization (data not shown). Thus, coll IV and gelatin coatings were used for culture experiments with CD34$^+$ cells. HUVEC proliferation (assessed by DNA synthesis) occurred on both gelatin-coated HAPs: a typical curve is shown in Fig. 1 for HAP2; tritiated thymidine was not retained on unseeded HAP2 used as control (not shown). Confluency was reached about 14 days after seeding. HUVEC formed a homogeneous monolayer on HAP (Fig. 2) and their morphological aspect was similar to that observed on coated coverslips.

3.2. Endothelialized HAP is permissive to CD 34$^+$ expansion

The endothelialized HAP was evaluated for its capacity to support the expansion and differentiation of CD34$^+$ cells in various conditions (Table I). Results indicate that (a) there was no expansion on uncellularized HAP and without cytokines, (b) there was no difference between coll IV or gelatin coating, (c) there was no difference between HAP1 or HAP2 porosity, (d) a moderate expansion was observed on endothelialized HAP in the absence of cytokines, (e) no (HAP1) or moderate (HAP2) expansion was observed in the presence of cytokines on unseeded HAP, (f) a major expansion was observed on endothelialized HAP in the presence of IL-1 and IL-3. Maximum expansion was obtained between days 7 and 14 of coculture. The viability of the expanded cells was always above 80% in the presence of cytokines and under 50% in the absence of cytokines. Moreover, it appeared by morphological analysis of cells expanded on endothelialized HAP2 that at day 7, 76% were myeloid cells, with 13% mature cells, whereas at day 14, 100% were myeloid cells with 33% mature cells. At day 21, almost total expanded cells were mature ones.

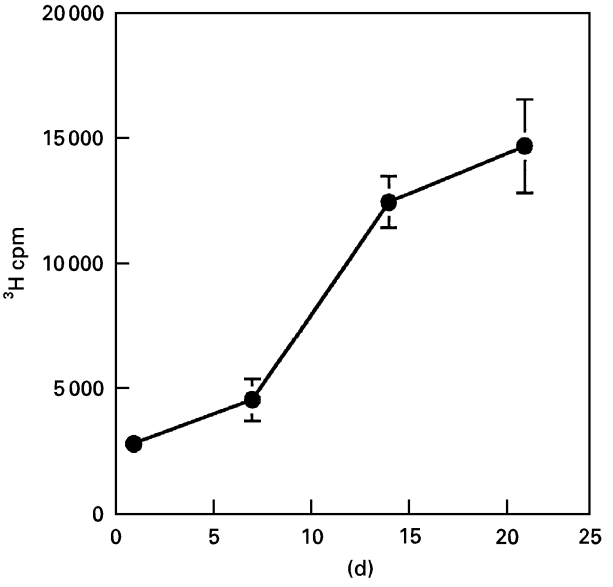

Figure 1 DNA synthesis analysed using tritiated thymidine incorporation as an index of HUVEC proliferation process on gelatin-coated HAP2 (●) (porosity 800–1000 μm). (Controls performed with unseeded material did not retain tritiated thymidine.)

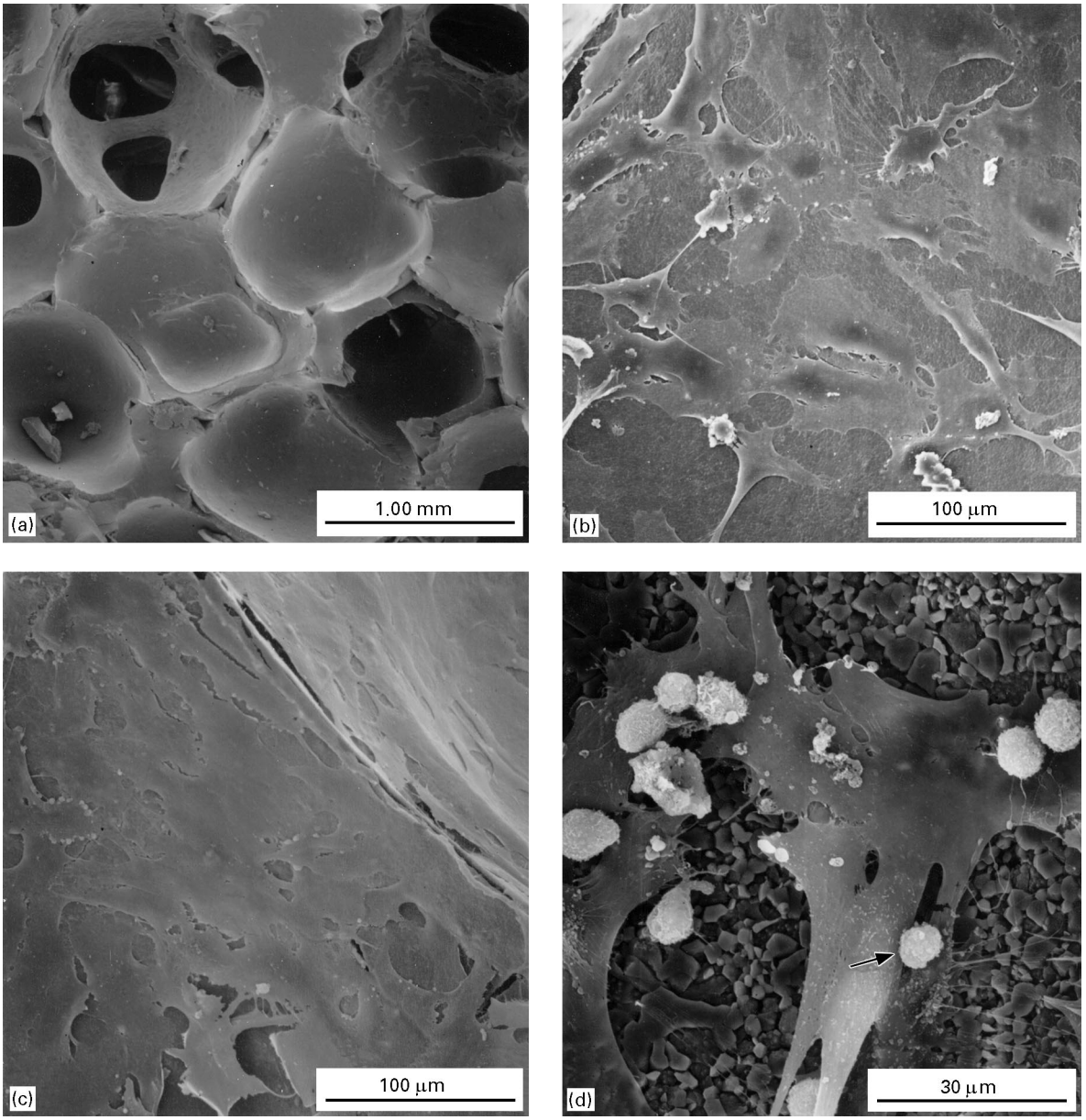

Figure 2 Scanning electron micrographs of gelatin-coated HAP2: without HUVEC (a); seeded with HUVEC 7 d before (b), 14 d before (c) coculture; (d) endothelialized and seeded with $CD34^+$ cells after 7 d coculture (arrow shows HPC adherent to HUVEC).

TABLE I Results of HPC expansion on endothelialized HAP in various conditions of porosity (400–700 μm = HAP1; 800–1000 μm = HAP2) and in different conditions of culture: presence (X) or absence (O) of IL-1 + IL-3 (IL); presence or absence of HUVEC; collagen IV (Coll) or gelatin (Gelatin) coatings. ND: not done. Values were calculated by dividing the total non-adherent cell numbers obtained at days 7, 14, 21, by the seeded cell number (5×10^3).

IL	HUVEC	Coll	Gelatin	Day 7 HAP1/HAP2	Day 14 HAP1/HAP2	Day 21 HAP1/HAP2
X	X	X		30/39	36/51	21/40
0	X	X		2/8.5	ND/ND	ND/ND
X	0	X		0.7/4.1	ND/ND	ND/ND
0	0	X		0/0.2	ND/ND	ND/ND
X	X		X	37/52	76.5/55	33/23
X	0		X	0.4/5.4	ND/ND	ND/ND

4. Discussion

The aim of our study was to investigate the potential of an endothelialized HAP as a cellularized support for the expansion of HPC. Recent reports indicate that EC can efficiently support the expansion and the differentiation of HPC without the need of exogenously added cytokines [6, 7, 14] and that addition of IL-1 and IL-3 to EC/HPC cocultures led to a dramatic improvement of the EC capacity to support the expansion of both total nucleated cells and colony-forming cells [6, 7]. Effort is currently being made to analyse the capacity of cellularized biomaterials to support

haematopoiesis, because they could improve the expansion of HPC and particularly the maintenance or expansion of the most primitive LTC-IC population. Compared with other cellularized matrices, such as glass capillaries [15], gelatin-modified glass microcarriers [5], HAP has potentially interesting features regarding the *in vitro* mimicking of haematopoiesis. The three-dimensional lattice structure of HAP may offer a more appropriate niche where cell/cell interactions could be favoured and be closer to their *in vivo* situation counterpart.

In the current study, we have demonstrated that HAP can be cellularized with HUVEC. Its precoating with gelatin, collagen, or fibronectin was found to be an essential prerequisite to the formation of an homogeneous monolayer. To analyse the functional ability of the endothelialized HAP to support haematopoiesis, we set out experiments with $CD34^+$ HPC in static culture conditions. Results show that endothelialized HAP is fully functional in supporting the proliferation and the differentiation of the HPC. The potential interest of an endothelialized HAP is that it is amenable to the development of a bioreactor that would allow a high cell density continuous perfusion system. The expanded cell population mainly belonged to the myelocytic lineage and the addition of IL-1 and IL-3 led to a major potentialization of the HAP capacity to support the proliferation of the HPC. These experiments also suggest that the immature HPC remained trapped within HAP while giving rise to a more mature progeny that exits from HAP microenvironment. Our results are consistent with a model in which the immature HPC remains closely associated with the feeder cells inside the matrix. Current experiments aim at characterizing this HUVEC-adherent population of HPC and to define their clonogenic potential.

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